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Genetic dissection of complete genomes of Type 2 PRRS viruses isolated in Denmark over a period of 15 years☆

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ABSTRACT

Type 2 Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) was first detected in Europe in 1996 co-incident with the introduction of a live attenuated vaccine. Since then, only limited ORF5 and ORF7 sequences of Type 2 PRRS viruses have been reported throughout Europe. In the present study, the genetic and antigenic diversity of 11 complete genomes and 49 ORF5 and 55 ORF7 nucleotide sequences obtained from 57 viruses in Denmark from 2003 to 2012 were examined. The genetic identity of the 11 complete genomes to the vaccine strain (Ingelvac PRRS MLV) ranged between 93.6 and 99.6% while the 49 ORF5 sequences examined were 94.0–99.8% identical to the vaccine strain. Among the Danish sequences, the pairwise nucleotide identity was 90.9–100% and 93.0–100.0% for ORF5 and ORF7, respectively. Analysis of the genetic region encoding NSP2 revealed high diversity among the Danish viruses with an 86.6–98.9% range in similarity. Furthermore, several of the sequenced viruses harbored deletions in the NSP2 coding region. Phylogenetic analysis in a global Type 2 PRRSV framework classified all Danish isolates to a single cluster (sub-lineage 5.1) which comprised strains closely-related to the Type 2 prototype isolate VR2332.

1. Introduction

A mystery swine disease causing reproductive failure in sows and severe pneumonia in piglets was first described in North America in the late 1980s and was a few years later also observed in Europe (Keffaber, 1989; Wensvoort et al., 1991). The etiological agent of the disease was found to be viral and finally named Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) based on the clinical signs (Terpstra et al., 1991; Wensvoort et al., 1991; Collins et al., 1992). Isolation and characterization of viruses obtained from the two continents indicated a pronounced degree of genetic heterogeneity with only 50–60% nucleotide identity (Wensvoort et al., 1991; Collins et al., 1992; Allende et al., 1999). Subsequently, PRRS viruses were divided into two major genotypes named Type 1 for the genotype initially described in Europe and Type 2 for the North American genotype (Allende et al., 1999). Nowadays, both genotypes circulate worldwide (Shi et al., 2010a).

PRRSV is an enveloped single-stranded positive-sense RNA virus, belonging to the *Arteriviridae* family within the order *Nidovirales* (Cavanagh, 1997). The genome is

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15–15.5 kb long and encodes 10 ORFs (Meulenberg et al., 1993; Wu et al., 2001; Firth et al., 2011; Johnson et al., 2011). ORF5, encoding the glycoprotein GP5, is one of the most variable genes and is therefore often used in phylogenetic analysis (Conzelmann et al., 1993; Key et al., 2001; Shi et al., 2010b). A serological survey performed in 1996 documented that Denmark was free of antibodies against Type 2 PRRSV prior to the introduction of a modified live vaccine (Botner et al., 1997; Madsen et al., 1998). Since then, Type 2 PRRSV infections have been sporadically reported throughout Europe, however, ORF5 and ORF7 sequences of European Type 2 PRRSVs are scarce. Apart from a Hungarian Type 2 PRRSV strain (Balka et al., 2008), all published European Type 2 viruses shared a high degree of identity to the PRRSV MLV vaccine strain (>98%) which is based on the VR2332 isolate. In 2006, China experienced the emergence of a highly pathogenic strain of PRRSV which had a unique genomic structure (Tian et al., 2007; An et al., 2010). The PRRSV epidemic in China affected more than 2,000,000 pigs with about 400,000 fatal cases. This abrupt occurrence of highly pathogenic PRRSV strains emphasizes the significance of monitoring the diversity of circulating strains around the world both in respect to the sensitivity and specificity of diagnostic tests as well as efficacy of available vaccines.

Today – more than 15 years after the initial introduction – Type 2 PRRSV still causes significant clinical problems in Danish herds. Previously sequenced Danish Type 2 PRRSV isolates were reported to have a high level of identity to the vaccine strain (Madsen et al., 1998; Storgaard et al., 1999). However, since only a few Danish PRRS viruses have been sequenced during the last 15 years, the diversity of circulating Type 2 viruses in Denmark and in the rest of Europe is unknown. This represents an important void especially since the export of Danish pigs to Eastern and central Europe is significant. For example, in 2012 Denmark exported more than 9 million live pigs whereas the import of breeding animals was much less ($n < 100$). Despite this limited import of live pigs, it is still possible that foreign PRRSV isolates may be introduced into Denmark by contaminated transport carriers or persons as recently seen in Sweden (Carlsson et al., 2009). Similarly, insight into PRRSV diversity in Denmark is of mutual interest for a range of countries. Accordingly, the main objective of the present study was to close the knowledge gap on the temporal diversity of circulating Type 2 PRRS viruses in Danish pigs. A comprehensive sequence analysis of ORF5 and its gene product, GP5, of Danish Type 2 PRRSV viruses isolated in the years 2003–2012 was performed and compared to the early introduced Danish Type 2 viruses and globally isolated Type 2 viruses. Additionally, for the first time, a thorough genetic and antigenic analysis of 11 complete genome sequences of Type 2 PRRSV isolated in Europe was conducted.

2. Material and methods

2.1. Sample material

Lung tissue, serum, oral fluid, and nasal swabs were obtained from 35 Danish swine herds in the years 2003–2012. A number of strains were propagated for one passage in Marc-145 cells using the general cell culture procedure (Kim et al., 1993).

2.2. RNA extraction

Total RNA was extracted from serum, nasal swabs, cell culture supernatant, and lung tissue. Lung tissue was prepared as a 5% homogenate in RLT buffer (QIAGEN) containing 1% β-mercaptoethanol (Sigma–Aldrich). RNA was extracted from lung homogenate and nasal swabs using RNeasy Minikit (QIAGEN) according to the manufacturer's instructions. Total RNA from serum and cell culture supernatant was purified using QIAamp Viral RNA Mini Kit (QIAGEN). Elution volume for both extractions methods was 60 μl. The RNA was stored at −80 °C until use.

2.3. Real time RT-PCR

Purified RNA was screened for PRRSV using a modification of the Primer Probe Energy Transfer RT-PCR (PriProET-RT-PCR) assay described by Balka et al. (2009).

2.4. cDNA synthesis and PCR amplification

Full-genome cDNA synthesis was performed by SuperScript® III First-Strand Synthesis System (Invitrogen) following the recommendations by the manufacturer except that the cDNA synthesis step was extended to 90 min. A poly(dT) RT-primer was used as cDNA primer (5′-CAG GAA ACA GCT ATG ACA CCT GAT CTC TAG AAA CGT $T(T)_{38}$-3′) (Nielsen et al., 2003). PCR amplification of ORF5 and ORF7 was carried out using the AccuPrime™ *Taq* DNA Polymerase High Fidelity Kit (Invitrogen). The PCR mixture was prepared as recommended by the supplier except that the amount of AccuPrime™ Taq High Fidelity was increased to 0.5 μl. The PCR amplification was conducted on a T3 thermo cycler (Biometra) with the following conditions: [94 °C for 15 s], 45 cycles: [94 °C 15 s, 55 °C for 30 s, 68 °C for 60 s] then finalize with 68 °C for 5 min and cool down to 4 °C. PCR primers for amplifying ORF5 and ORF7 were from Oleksiewicz et al. (1998). PCR products were analyzed on 2% agarose gels (E-gels, Invitrogen).

Long range PCR amplification was performed using the full-genome cDNA as template with AccuPrime™ Taq High Fidelity kit as described by Kvisgaard et al. (2013).

2.5. Cycle sequencing and next generation sequencing

PCR products of the ORF5 and ORF7 amplifications were sequenced by cycling sequencing using the Sanger method (Sanger et al., 1977) with the ORF5 and ORF7 primers used as sequencing primers (Sanger et al., 1977). In total, 39 ORF5 sequences (accession no. KC506625; KC506628-30; KC506632-35; KC506637-41; KC506643-59; KC506661-662; KC506665; KC506667-69; KC506671-72; KC577601) and 45 ORF7 sequences (accession no. KC506674; KC506677-79; KC506681-84; KC506686-90; KC506692-712; KC506714-15; KC506718; KC506720-22; KC506724-26; KC577602-3) were generated.

Full genome sequences were generated by next generation sequencing technologies (NGS) using long range PCR amplicons covering the full genome of PRRSV in two or four fragments as templates. For a detailed description of the procedure and next generation sequencing see Kvisgaard et al. (2013). In short, equimolar concentration of the PCR amplicons covering the full genome of PRRSV were prepared for sequencing on Roche/454 Genome Sequencer FLX + Titanium (LGC Genomics GmbH, Berlin, Germany) and Ion Torrent GPM sequencer (DTU Multi-Assay Core (DMAC), Kgs. Lyngby, Denmark). Additionally, full length sequences of two Danish viruses (DK-2010-10-2-1 and DK-2010-10-7-1) were obtained using the 454 GS Jr. platform. DNA template for these two isolates was generated using a random PCR method previously described (Van Doorsselaere et al., 2011).

2.6. Sequence analysis

Data analysis of ORF5 and ORF7 sequences obtained from the cycle sequencing was carried out using the commercial software CLC Main Workbench v. 6.6.2 (CLC BIO, Arhus, Denmark). Contigs of ORF5 and ORF7 were produced from assembling the raw data obtained from cycle sequencing against the reference sequence VR2332 (PRU87392). Nucleotide and amino acid sequences were aligned using MUSCLE (MUltiple Sequence Comparison by Log-Expectation) (Edgar, 2004). The ORF5 sequences were classified based on a globally representative ORF5 phylogenetic framework (Shi et al., 2010b). The ORF5 phylogenetic tree of the newly sequenced Danish isolates and reference sequences was constructed using a Bayesian Markov chain Monte Carlo (BMCMC) method implemented in MrBayes v3.2 (Ronquist et al., 2012) under settings previously described (Shi et al., 2010b).

Mapping of reads obtained from full genome sequencing was performed by the Burrows-Wheeler aligner (BWA) using the bwasw algorithm for Roche 454 FLX and Ion Torrent PGM sequencer data (Kvisgaard et al., 2013). Amino acid sequences were predicted from the nucleotide sequences using CLC Main Workbench v. 6.6.2. Nucleotide and amino acid sequences were aligned using MUSCLE (MUltiple Sequence Comparison by Log-Expectation). The phylogenetic tree constituting the complete genomes were constructed from Neighbor Joining Algorithm with Bootstrap: 1000 replicates (CLC Main Workbench v. 6.6.2, CLC BIO, Arhus, Denmark).

Potential N-glycosylation sites were determined for GP2, GP3, GP4, and GP5 using NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/). Using a neural network, N-glycosylation sites were selected when potential above the threshold 0.5 was reached (http://www.cbs.dtu.dk/services/NetNGlyc/output.php).

3. Results

3.1. Analysis of ORF5 and ORF7 sequences

A total of 49 ORF5 and 55 ORF7 nucleotide sequences were obtained from 57 viruses collected in the years 2003–2012. The lengths of the ORF5 and ORF7 sequences were 603 and 372 nucleotides, respectively. The viruses DK-2011-30-3-13, DK-2011-30-3-15, and DK-2011-30-3-20 collected simultaneously from the same pig herd, were almost identical and had a 3 nucleotide deletion in ORF5 (corresponding to residues114–116 in Ingelvac PRRS MLV). This deletion was also present in two non-European viruses, one from Thailand and one from the USA (THA-2002-02SP3 and US-ISU3927) however these isolates shared only 86.8% and 92.3% identity in ORF5 to these three Danish sequences. Among the Danish sequences, the pairwise nucleotide identity was 90.9–100% and 93.0–100.0% for ORF5 and ORF7, respectively. The pairwise nucleotide identity of the Danish sequences to the Ingelvac PRRS MLV vaccine strain was in the range 94.0–99.8% and 94.6–100% in ORF5 and ORF7, respectively. Phylogenetic analysis of ORF5 in a globally representative context (Shi et al., 2010b) showed all the Danish viruses to be closely related to the cluster of VR2332-like or vaccine-like strains i.e. sub-lineage 5.1 (Fig. 1). Similar comparisons performed exclusively among Danish isolates showed a maximum nucleotide diversity of 9.1%. Pairwise nucleotide comparisons based on a partial ORF5 region (nt91-522) between Danish Type 2 PRRS viruses and two Hungarian Type 2 PRRS viruses from 2005 to 2006 (Balka et al., 2008) showed only 84.2–88.2% identity (data not shown).

3.2. Antigenic analysis of the amino acid sequence of the major glycoprotein 5, GP5

Examination of the deduced amino acid sequences of ORF5, GP5, showed that 144 positions out of 200 were conserved in all of the 49 Danish sequences (72% conserved sites). Twenty-four positions varied in more than one virus, and 7 positions varied in more than 10 viruses. The most variable position was at aa34 where 34 viruses varied. Six different amino acid residues were seen at this site. Interestingly, position aa34 is a putative N-glycosylation site in Type 2 PRRS viruses. The cysteine residue at position aa48 thought to be involved in heterodimer formation with the M protein (Mardassi et al., 1996) was conserved in all the Danish sequences. The decoy epitope (A/VLA/VN motif) at position aa27–30 (Ostrowski et al., 2002) is located in a hypervariable region of GP5 and this motif was conserved in the majority of the Danish viruses. The neutralizing epitope of GP5 located in the region aa37–45 (SHL/FQLIYNL) is known to be highly conserved, which also was seen for the Danish viruses (Ostrowski et al., 2002).

Potential N-glycosylation sites were observed at seven different positions: N30, N32, N33, N34, N35, N44, and N51. Using a neural network, N-glycosylation sites were predicted to be utilized if the threshold was above 0.5. The result of these network predictions, revealed 8–9 different putative glycosylation patterns among the Danish viruses. N-glycosylation sites variations were located mainly between aa30–35 near the highly variable region, including the highly variable site at position aa34 as mention previously. The N44 and N51 N-glycosylation sites were conserved in all but one sequence, where N44->D44 but the N51 glycosylation site was conserved in this virus (DK-2012-10-1-5). The most common N-glycosylation pattern was N30, N33, N44, and N51 which 34 viruses harbored,

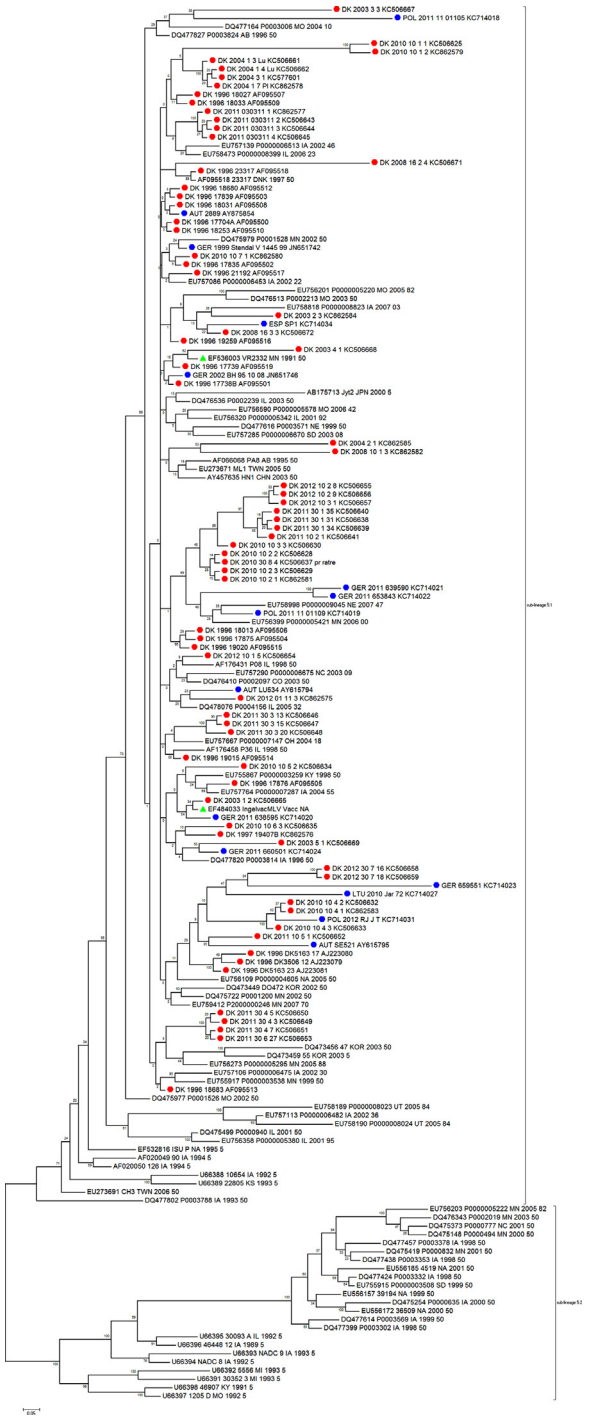

Fig. 1. Phylogenetic analysis of the Danish ORF5 sequences in a globally representative context. All the Danish sequences are marked with a red dot, all European sequences are marked with a blue dot, and the vaccine strain and the Type 2 protogenotype VR2332 are marked with green triangles. All the Danish ORF5 sequences were closely related to the cluster of VR2332-like or vaccine-like strains i.e. sub-lineage 5.1. The tree was constructed using a BMCMC method implemented in MrBayes v3.2.

however, in 14 of these sequences N30 did not reach the potential threshold of 0.5 and therefore was not predicted to be N-glycosylated by the neural network.

3.3. Genetic analysis of 11 complete Danish PRRSV Type 2 genomes

Eleven complete genomes of Type 2 PRRSV (Kvisgaard et al., 2013) isolated from Danish pig herds in the years 1997–2012 were aligned and analyzed. The genome lengths varied from 15,396 to 15,408 nucleotides (excluding the Poly(A) tail) and the pairwise nucleotide identity among the Danish viruses was 90.6–99.3% and they were 93.6–99.6% identical to the vaccine strain Ingelvac PRRS MLV (Table 1). DK-2008-10-1-3 and DK-2010-10-1-2 were clearly different from the majority of Danish isolates in that the pairwise nucleotide identities to the other Danish sequences were 92.8–95.3% and 91.1–93.6%, respectively. These two isolates were only 90.6% identical. Furthermore, DK-2010-10-1-2 shared only 93.6% nucleotide identity to Ingelvac PRRS MLV vaccine making it the most diverse of the Danish viruses at the genetic level. A phylogenetic tree was constructed with the complete Danish sequences and Type 2 sequences isolated in North America and China (Fig. 2). Despite the fact that the Danish isolates varied up to 10% of genomic sites, they all grouped together in a cluster which also included the vaccine strain, VR-2332 and three other US strains isolated in the mid-1990s. This grouping largely mirrored the phylogenetic clustering (lineage 5) described earlier using ORF5 only.

The pairwise nucleotide and deduced amino acid identity of the non-structural proteins (NSP/nsp) and structural proteins of the Danish viruses were examined. The average similarity of the different ORFs ranged from $94.9 \pm 2.9\%$ for ORF1a to $97.8 \pm 1.5\%$ for ORF6. The low average similarity of ORF1a was mainly due to the low similarity in the coding regions of NSP1β and NSP2 which were in the range of 85.1–99.3% and 86.6–98.9%, respectively. The similarity of the protein products, nsp1β and nsp2, was also low (80.8–99.5% and 94.0–98.2%), thus indicating that most mutations in this region are non-synonymous. For a complete overview of the pairwise identities of the different ORFs see Supplementary Table 1.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetmic.2013.09.023.

3.4. Examination of the non-structural protein 2 (nsp2) of the Danish Type 2 PRRS viruses

Fig. 3 shows an alignment of the deduced nsp2 amino acid sequences of 11 Danish viruses together with three North American isolated Type 2 PRRS viruses reported to be highly virulent (Han et al., 2006; Gauger et al., 2012) and three Chinese PRRSV Type 2 viruses, one high pathogenic (GDBY1), one low pathogenic (GDQJ), and one (BJ-4) very similar to Ingelvac PRRS MLV (99.7% nucleotide identity) (Yan et al., 2007; Li et al., 2010). The virus, BJ-4, has also been used for B-cell epitope identifications in nsp2 (Yan et al., 2007). Alignment of

Table 1
Overview of the 11 Danish Type 2 PRRSV complete genomes.

Virus	Accession no.	Collection year	Clinical signs (herd)	Sequencing material	Genome length[a]	Nt identity%[b]
DK-1997-19407B	KC862576	1997	Stillborn piglet[c]	Marc-145 isolate	15,396	99.4
DK-2003-2-3	KC862584	2003	–	Marc-145 isolate	15,408	96.1
DK-2004-2-1	KC862585	2004	–	Marc-145 isolate	15,408	96.0
DK-2004-1-7-Pl	KC862578	2004	–	Marc-145 isolate	15,408	99.4
DK-2008-10-1-3	KC862582	2008	Reproductive failure	Lung homogenate	15,408	95.4
DK-2010-10-1-2	KC862579	2010	Respiratory	Lung homogenate	15,342	93.6
DK-2010-10-7-1	KC862580	2010	Respiratory	Marc-145 isolate	15,399	98.8
DK-2010-10-2-1	KC862581	2010	Respiratory	Marc-145 isolate	15,399	99.6
DK-2010-10-4-1	KC862583	2010	Respiratory	Lung homogenate	15,399	98.1
DK-2011-030311-1	KC862577	2011	High fever	Lung homogenate	15,408	99.3
DK-2012-01-11-3	KC862575	2012	Respiratory/ reproductive failure	Lung homogenate	15,399	98.3

[a] Excluding the Poly(A) tail.
[b] Pairwise nucleotide identity to Ingelvac PRRS MLV (EF484033).
[c] This virus was obtained from a non-vaccinated herd about 6 months after the Danish vaccination program had started in the fall 1996.

the predicted amino acid sequence of nsp2 of the Danish viruses with the vaccine strain revealed that several of the Danish viruses harbored deletions resulting in variation of the length of nsp2 from 1174 to 1096 amino acids residues. One virus harbored a 4 amino acid deletion following the amino acid at position 793 and five other viruses harbored a 3 amino acid deletion at the position corresponding to aa593–595 in nsp2 of Ingelvac PRRS MLV. One of the viruses harboring the 3 amino acid deletion was the virus DK-2010-10-1-2 and this virus also had a 19 amino acid deletion corresponding to the position aa498–516 in Ingelvac PRRS MLV nsp2. Thus, this virus encoded the shortest nsp2 of the Danish viruses with an amino acid length of 1174 residues. All the deletions observed in the Danish viruses were located at the hyper variable region between aa150–850 of nsp2. The nsp2 protein is known to be the most diverse protein of all the proteins encoded by PRRSV, nevertheless, the cysteine protease domain (PL2) located in the region aa46–146 of nsp2 is known to be conserved within the same genotype. Indeed, this region was also highly conserved in the Danish viruses with only 17 substitution sites out of 101positions while the North American and Chinese viruses had only 16 and 9 substitution sites, respectively, in comparison to the Ingelvac PRRS MLV nsp2 sequence. The amino acid residues forming the putative catalytic triad C54, D88, and H123 (Han et al., 2009), were conserved in all the Danish viruses as well as in the American and Chinese viruses. All 6 putative B-cell epitopes identified by Yan et al. (2007) are highlighted in Fig. 3. Epitope sites SP1, SP2, and SP4 were highly conserved among the Danish viruses, whereas epitope sites SP5, SP6, and SP8 showed a high proportion of variable sites (60%, 64%, and 69%, respectively).

Comparison of the Danish nsp2 sequences to the vaccine strain showed high similarity for 7 of the viruses (DK-1997-19407B, DK-2004-1-7-Pl, DK-2010-10-2-1, DK-2010-10-4-1, DK-2010-10-7-1, DK-2011-030311-1, and DK-2012-01-11-3) with 96.2–99.3% amino acid identity and lower similarity for 4 viruses (DK-2003-2-3, DK-2004-2-1, DK-2008-2-1, and DK-2010-10-1-2) with amino acid identity in the range of 89.0–92.5%.

3.5. Antigenic analysis of the minor glycoproteins, GP2, GP3, and GP4

From the pairwise amino acid examinations, GP3 was shown to be the most diverse structural protein among the Danish Type 2 viruses with amino acid identity of 90.9–99.2%. Linear B-cell epitopes have been identified in all 3 minor glycoproteins using Pepscan technologies using the sequence of the virus strain NVSL 97-7895 (de Lima et al., 2006). The GP2 comprised two putative linear B-cell epitopes at amino acid positions aa41–55 and 121–135, respectively. These regions were highly conserved among the Danish viruses; however, the epitope located at the region aa41–55 harbored 4 substitutions sites compared to the NVSL 97-7895 strain. For GP3, 4 overlapping consecutive linear B-cell epitopes have been identified (aa61–75, aa71–85, aa81–95, and aa91–105) (de Lima et al., 2006). Even though GP3 was the most diverse structural protein among the Danish Type 2 viruses, the 4 epitope sites were all highly conserved with only 8 substitution sites distributed throughout all 4 epitope regions (aa61–105) when compared to the NVSL 97-7895 strain GP3 sequence (Supplementary Fig. 1). The putative epitope site in GP4 at position aa51–65 was not conserved among the Danish viruses with 10 substitution sites out of a possible of 15 (Supplementary Fig. 2).

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetmic.2013.09.023.

Examination of potential N-glycosylation sites revealed 2 putative N-glycosylation sites at N178 and N184 in GP2, 7 putative N-glycosylation sites (N29, N42, N50, N131, N152, N160, and N195) in GP3, and 4 putative N-glycosylation sites (N37, N84, N120, and N130) in GP4. The result of nine neural networks predicted all putative N-glycosylation sites in GP2 and GP4 to be N-glycosylated however N37 in GP4 did not score a unanimous vote from the jury network to be N-glycosylated. For GP3 all 7 putative N-glycosylation sites were predicted to be N-glycosylated in all the Danish viruses except for virus DK-2008-10-1-3 where the potential at this position did not reach the threshold of 0.5. A unanimous vote from the jury predicted N-glycosylation at N195 however this position is

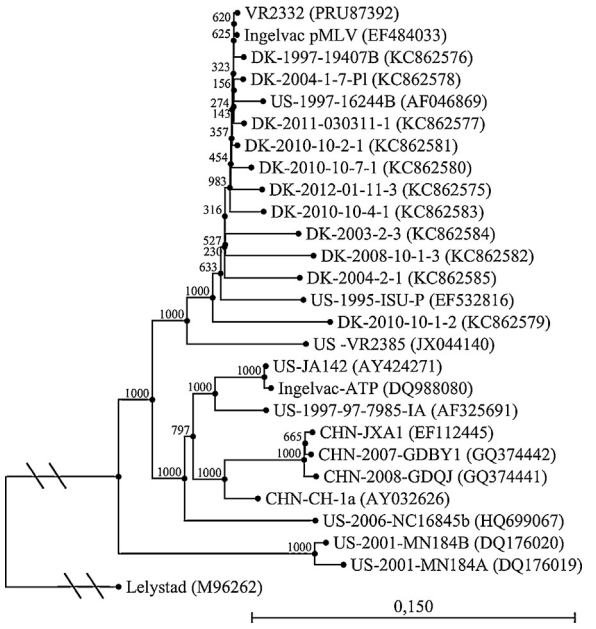

Fig. 2. Phylogenetic analysis of 11 Danish complete PRRSV Type 2 genomes and 10 complete genomes of viruses isolated in China and North America. The tree was constructed by the neighbor-joining method using CLC Main workbench (v. 6.6.2). Bootstrap values were calculated from 1000 replicates and shown on branches. The scale bar represents 15% nucleotide change. PRRSV Type 1 Lelystad is used as outgroup (set root above node). CHN = China, DK = Denmark, US = United States of America.

believed to be located in the transmembrane region of GP3, hence unlikely to be glycosylated (Dokland, 2010).

The cysteine residues important for protein folding and function was 100% conserved in all the minor structural proteins of the examined Danish viruses.

4. Discussion

We report here for the first time the complete genome sequences obtained from Type 2 PRRS viruses isolated in Europe. With the 11 complete genomes sequenced from viruses isolated in the years 1997–2012 (Kvisgaard et al., 2013), the evolution of Type 2 PRRSV since its introduction 15 years ago in Denmark is now well described. The virus DK-1997-19407B was isolated from a non-vaccinated herd about 6 months after the Danish PRRSV Type 2 vaccination program first started in the fall of 1996 (Nielsen et al., 2001). The complete genome of this virus was 99.4% identical to the vaccine strain and to two other Type 2 viruses isolated in the US in 1995 and 1997, respectively. These findings support epidemiological data indicating that Type 2 PRRSV was likely introduced into Denmark by the MLV vaccine.

The sudden emergence of more pathogenic PRRS viruses in North America (Han et al., 2006; Gauger et al., 2012), Asia (Tian et al., 2007; An et al., 2010) and Eastern Europe (Karniychuk et al., 2010; Weesendorp et al., 2013; Morgan et al., 2013) emphasize the importance of monitoring the genetic diversity of PRRSV. Despite this obvious need, there has been only a limited focus on the genetic diversity of Type 2 PRRSV in Europe (Stadejek et al., 2013). Thus the perception has been that all Type 2 viruses in Europe are closely related to the attenuated vaccine strain and are only minor contributors to clinical PRRS in Europe. In Hungary, however, where the Ingelvac PRRS MLV vaccine has not been used (Balka et al., 2008), two Type 2 PRRS viruses were isolated in 2005 and 2006 that shared higher level of identity to a Canadian strain than to any Type 2 MLV strains, indicating that they may have been introduced by other sources than by vaccination (Balka et al., 2008). Pairwise nucleotide comparison of the partial ORF5 sequences of the Hungarian viruses to the 49 Danish viruses examined in our study revealed that they only shared 84.2–88.2% nucleotide identity and therefore suggests that they did not originate from Denmark, despite the use of Danish boar semen in Hungarian herds.

The majority of the 11 complete genomes showed high level of similarity to the vaccine strain with pairwise nucleotide identity of 98.1–99.6%. This similarity correlated well with the diversity in ORF5 of the Danish viruses where all the ORF5 sequences clustered along with the vaccine strain in lineage 5 (Shi et al., 2010b). Interestingly, four viruses from 2003, 2004, 2008, and 2010 showed a higher level of diversity to the vaccine strain with 93.6–96.1% complete genome similarity, however, they still clustered within the lineage 5 in the phylogenetic analysis. Based on the ORF5 nucleotide sequences obtained from all Danish viruses isolated between 2003 and 2012, the diversity of the Danish viruses to the vaccine strain was in the range of 94.0–99.8%, which is a greater range than the diversity of most European Type 2 viruses (>98% similarity to the vaccine strain) (Greiser-Wilke et al., 2010; Stadejek et al., 2013). The diversity among 1400 global Type 2 ORF5 sequences retrieved from a database was 3.5% (Shi et al., 2010b) which also is less than the diversity of 9.1% seen among the Danish ORF5 sequences. The reason for this difference is not clear, but it indicate that the selection pressure on Danish PRRSV strains is different than on other Type 2 isolates maybe because it originated from an attenuated field strain. Most of the Danish samples included in the present study originated from herds with suspected acute outbreak of PRRSV. It is therefore very unlikely that these herds vaccinated against PRRSV at the time of submission, however, there are no reliable makers that can be used to discriminate between the vaccine strains and circulating Type 2 field strains.

Notwithstanding insights based on ORF5, the examination of limited genomic regions may not be sufficient for a more complete understanding of PRRSV sequence heterogeneity, as illustrated by the non-structural protein coding regions NSP1β and NSP2 of the Danish viruses that showed a very broad range of identity to the vaccine strain of 85.1–99.3% and 86.6–98.9%, respectively.

DK-2010-10-1-2 was the virus with the shortest genome constituting 15,342 nucleotides resulting from a discontinuous deletion in the coding region of NSP2 of 57 plus 9 nucleotides (corresponding to 19 plus 3 amino acids in the deduced amino acid sequence of nsp2, Fig. 3). The consecutive 9 nucleotide deletion was also seen in 4 other viruses examined in this study. Although some of these viruses were sequenced from RNA extracted from cell

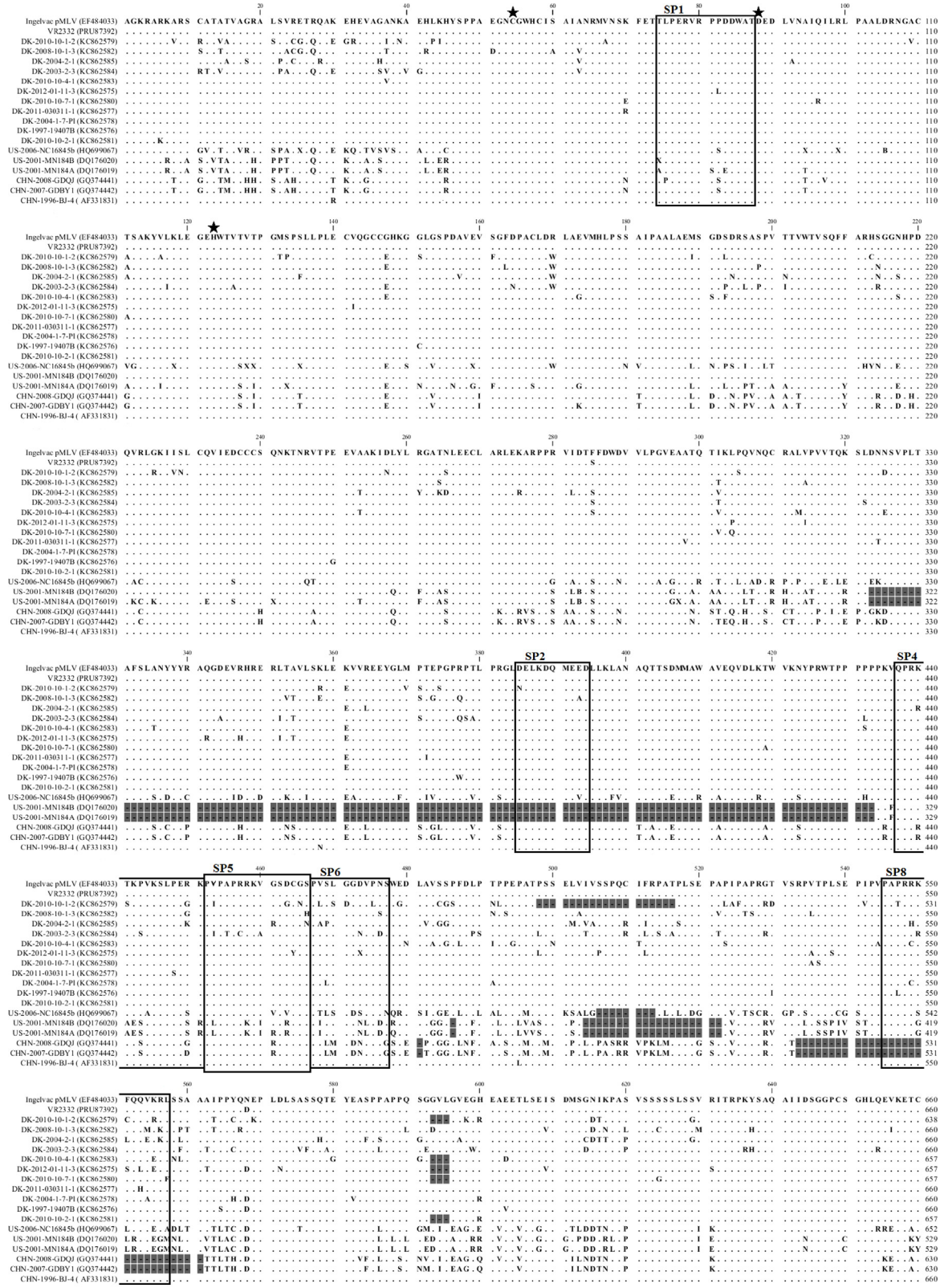

Fig. 3. Amino acid alignment of nsp2. Putative B-cell epitopes (Yan et al., 2007) are highlighted in black boxes. The amino acid residues forming the putative catalytic triad C54, D88, and H123 (Han et al., 2009) are marked with black stars. Deletion sites are marked with gray rectangles.

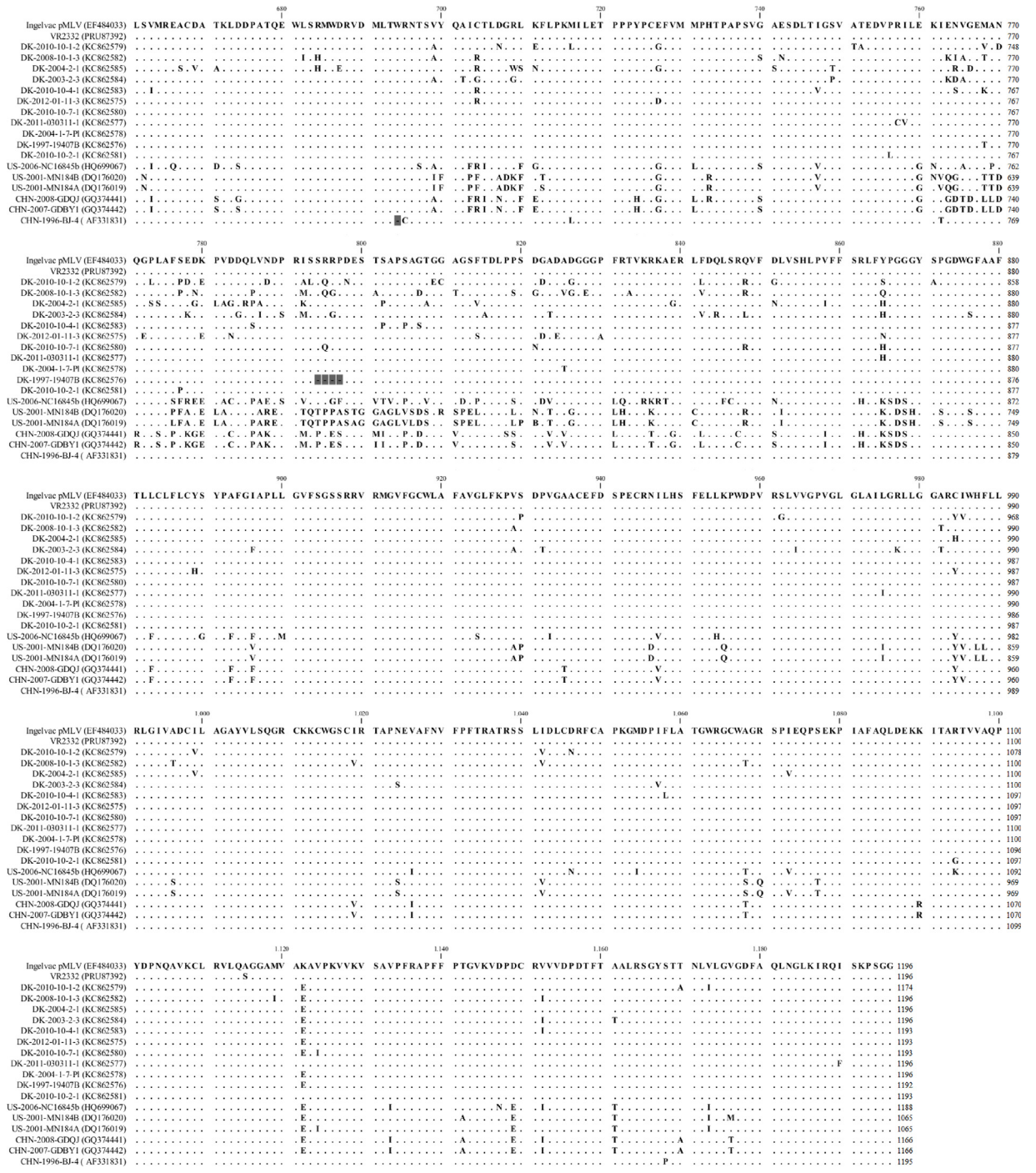

Fig. 3. (*Continued*).

culture supernatant, the deletion is naturally occurring since it was also found in partial NSP2 sequences obtained from the corresponding primary material (data not shown). Deletions are often seen in the NSP2 coding part of the PRRSV genome (Ropp et al., 2004; Han et al., 2006), and also in high virulent strains such as the MN184A and MN184B viruses and all the high pathogenic viruses isolated in China during the outbreak in 2006–2009 (An et al., 2010). Based on these findings, one might presume that deletions in NSP2 were linked to high virulence of the virus. However, a low pathogenic virus, GDQJ (GQ374441), isolated in 2008 was shown to harbor the exact same deletion as the high pathogenic Chinese viruses (Li et al., 2010). Additionally, using chimeric viruses, the NSP2 deletion in the 2006 Chinese outbreak of PRRS was shown not to be linked to virulence (Zhou et al., 2009). In this

sense, the deletions found in NSP2 of the Danish viruses can be regarded as an 'epidemiological marker' and not as a marker of virulence.

The nsp2 is the viral protein containing the highest frequency of B-cell epitopes (Oleksiewicz et al., 2001; de Lima et al., 2006; Yan et al., 2007) and the humoral antibody response to nsp2 is more pronounced than toward any other PRRSV protein. However, most of the antibodies are non-neutralizing (Han et al., 2007; Johnson et al., 2007; Brown et al., 2009). Analysis of the Danish viruses showed that epitope sites SP1, SP2, and SP4 identified by Yan et al. (2007) were highly conserved among the Danish viruses, whereas epitope sites SP5, SP6, and SP8 showed a high proportion of variable sites of 60%, 64%, and 69%, respectively (Fig. 3). de Lima et al. (2006), identified 18 B-cell epitopes in nsp2 of a North American virus (NVSL 97-7895, acc. no. AY545985), but none of these epitopes were conserved in the Danish nsp2 sequences probably as a consequence of the high level of amino acid variation of nsp2 (amino acid comparison of the North American nsp2 sequence to the Danish nsp2 sequences were 77.5–82.7% identity). The North American virus, NVSL 97-7895 was also used for screening of linear B-cell epitopes in the structural proteins and the epitopes identified in GP2 and GP3 was highly conserved among the Danish viruses and to the North American strain although the overall amino acid variation was high in all three minor glycoproteins compared to the Danish viruses. The B-cell epitope identified in GP4 at position aa51–65 were highly variable with 10 substitutions out of 15 possible. This fits well with previous findings that this protein only reacted with a small fraction of tested post-infection sera (de Lima et al., 2006). Taken together these results indicated that some – but not all – epitopes present in the nonstructural and structural proteins of Type 2 PRRSV are prone for changes – which again indicate that antibodies generated against some epitopes may have no impact on virus survival. On the other hand, the finding that some of the epitopes were conserved despite decades of drifts may actual indicate that conservation of these sites favor viral survival maybe by directing immunological attention to sites that are not harmful for the virus. These considerations emphasize that comparisons of complete genomes provides valuable information on conserved and variable regions and thereby can reveal new targets for immune intervention, the design of new vaccines and for the development of diagnostic tests.

The N-glycosylation of GP5 may be critical for proper functioning of the protein. N-glycosylation, in general, is important for correct folding, targeting, and biological activity of proteins (Ansari et al., 2006). In this study, potential N-glycosylation sites were observed at seven different positions in GP5. The result of a neural networks predictions lead to 8–9 different putative glycosylation patterns among the Danish viruses, with the N-glycosylation of N30, N33, N44, and N55 the most abundant pattern. However, even though the N-glycosylation motif at N30 was present in 14 sequences it was not predicted to be N-glycosylated. The position N30 is located in the C-terminal part of the putative signal peptide and glycosylation at this position may not have any influence on the mature protein, but may contribute to other biological functions such as immune evasion. The N-glycosylation at position N44 has previously been shown to be important for infectious virus production (Ansari et al., 2006) and only one of the glycosylation patterns found among the Danish GP5 sequences failed to predict this site to be glycosylated.

Of the 11 complete genomes examined in this study, all of the putative N-glycosylation motifs in the minor glycoproteins GP2, GP3, and GP4 were present and predicted to be N-glycosylated which strongly indicates that the glycosylation of these motifs are important for the functioning of the respective proteins.

In conclusion, this study presents for the first time in-depth analysis of complete genomes obtained from Type 2 PRRS viruses isolated in Europe. From the complete genomes it was revealed that there was an overall high diversity between the Danish viruses (90.6–99.3%) and one virus was only 93.6% similar to the vaccine strain. These results indicate that there has been pronounced genetic drifts of the Danish Type 2 PRRSVs but the data do not support multiple introductions of different Type 2 strains into Denmark which fits well with a limited import of living animals and semen into Denmark. Lastly, Type 2 viruses in Denmark seem to induce more severe clinical signs compared to other European countries but share relatively close evolutionary relationship in ORF5 to Type 2 viruses found in other European countries. This could indicate that the genetic determinants of viral virulence lie outside ORF5 and emphasize that the generation of complete genome sequences of European Type 2 isolates should be prioritized.

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